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(54) Title: IMPROVED METHODS FOR TRANSPLANTATION USING MODIFIED CELLS AND T CELL INHIBITORY AGENTS

(57) Abstract

Improved methods for inhibiting rejection of transplanted cells in an allogeneic or xenogeneic recipient subject are described. The methods involve altering at least one antigen on the surface of a donor cell prior to transplantation to reduce the immunogenicity of the cell in a recipient subject. Preferably, an MHC class I antigen on a donor cell is altered by contacting the cell with a molecule which binds to the antigen, such as an antibody or fragment or derivative thereof. According to the methods of the invention, a recipient subject is also treated with an agent which inhibits T cell activity in the subject. A preferred agent which inhibits T cell activity in the recipient subject is an immunosuppressive drug, such as cyclosporin A. Alternatively, antibodies which deplete T cells or inhibit T cell proliferation in the subject can be used. Typically, following administration of a modified donor cell to an allogeneic or xenogeneic recipient subject, an immunosuppressive drug is administered to the recipient subject for a sufficient time to induce tolerance to the donor cells.

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IMPROVED METHODS FOR TRANSPLANTATION USING MODIFIED CELLS AND T CELL INHIBITORY AGENTS

Background of the Invention

5 A number of diseases are treated by the transplantation of tissue donated by other human (allografts) or obtained from animals (xenografts). Examples of such diseases include Parkinson's disease, which can be treated by transplantation of neural cells, and insulin-dependent diabetes, which can be treated by transplantation of insulin-secreting pancreatic islet cells. While the transplanted cells may have the capacity to perform the desired function 10 (e.g., secretion of insulin in response to the rising levels of glucose), the graft will soon fail as a result of immunological rejection. Shortly after transplantation, cells of the immune system of the recipient recognize the allogeneic or xenogeneic cells as foreign and proceed to attack the graft through both humoral and cellular routes. Allogeneic or xenogeneic cells are initially recognized by the recipient's immune system through antigenic determinants 15 expressed on the surface of the cells. The predominant antigens recognized as "non-self" are the major histocompatibility complex class I and class II antigens (MHC class I and class II). MHC class I antigens are expressed on virtually all parenchymal cells (e.g., pancreatic islet cells). In contrast, MHC class II antigens are expressed on a limited number of cell types, primarily B cells, macrophages, dendritic cells, Langerhans cells and thymic epithelium. The 20 interaction of foreign MHC antigens with the T cell receptor on host T cells causes these host cells to become activated. Following activation, these T cells proliferate and induce effector functions which lead to cell lysis and destruction of the transplanted cells.

For transplantation to be a viable therapeutic option, approaches are needed to inhibit rejection of transplanted cells by the immune system of the recipient. One method for 25 inhibiting this rejection process is by administration of drugs that suppress the function of the immune system. Drugs such as cyclophosphamide and cyclosporin can inhibit the actions of the immune system and thus allow graft acceptance. However, these drugs generally need to be administered to a graft recipient permanently (i.e., lifelong) and their use results in generalized immunosuppression which leaves the recipient susceptible to infection and tumor 30 growth. Additionally, administration of immunosuppressive drugs is often accompanied by other serious side effects such as renal failure and hypertension. The requirement for life-long administration of immunosuppressive drugs in transplant recipients illustrates the need for better methods for transplanting cells such that rejection of the cells by the recipient's immune system is inhibited.

35 It has been shown that it is possible to alter an antigen on the surface of a cell to be transplanted prior to transplantation to "mask" the antigen from normal recognition by cells of the recipient's immune system (see Faustman & Coe (1991) *Science* 252:1700-1702 and WO 92/04033). For example, MHC class I antigens on transplanted cells can be altered by contacting the cells with a molecule which binds to the antigen, such as an antibody or

fragment thereof (e.g., a F(ab')₂ fragment) prior to transplantation. This alteration of MHC class I antigens modifies the interaction between the antigens on the cells and T lymphocytes in the recipient following transplantation, to thereby inhibit rejection of the transplanted cells. Additional methods for inhibiting rejection of an allograft or xenograft following
5 transplantation in a host are needed.

Summary of the Invention

This invention pertains to methods for transplanting cells into an allogeneic or xenogeneic recipient such that rejection of the cells by the recipient is inhibited. The
10 methods of the invention involve modification of donor cells prior to transplantation to reduce the immunogenicity of the cells in a recipient and treatment of the recipient with an agent which inhibits T cell activity in the recipient. In particular, this invention features treatment of donor cells to modify surface antigens prior to transplantation in conjunction with treatment of the recipient with a T cell inhibitory agent. As a result of the combined
15 treatment, an improved method for transplantation which is more successful than either treatment alone is provided.

One aspect of the invention pertains to methods for transplanting cells into an allogeneic or xenogeneic recipient whereby cells are treated prior to administration to alter at least one antigen on the cell surface and inhibit rejection of the cell by a recipient. The
20 antigen(s) on the surface of the cells to be altered is one which is capable of stimulating an immune response against the cell in the recipient. Alteration of the antigen(s) prior to transplantation modifies an interaction between the antigen and a hematopoietic cell in the recipient (e.g., a T lymphocyte), thereby inhibiting an immune response against the cell in the recipient. An antigen on the surface of a cell can be altered prior to transplantation by
25 contacting the cell *in vitro* with a molecule which binds to the antigen. In one embodiment, the molecule which binds to the antigen is an antibody, or fragment or derivative thereof, which binds to the antigen but does not activate complement or induce lysis of the cell. A preferred antibody fragment is an F(ab')₂ fragment. Alternatively, the molecule is a peptide or derivative thereof (e.g., a peptide mimetic) which binds the antigen and interferes with an
30 interaction with a hematopoietic cell. In a preferred embodiment, the antigen on the cell surface which is altered is an MHC class I antigen. Preferred antibodies which can be used to alter MHC class I antigens on the surface of cells include the monoclonal antibodies W6/32 and PT85, or fragments or derivatives thereof, or other antibodies which bind to the same epitopes recognized by the W6/32 and PT85 antibodies. Other cell surface antigens which
35 can be altered include adhesion molecules, such as ICAM-1, ICAM-2 and LFA-3.

Another aspect of the invention pertains to methods for transplanting cells in which T cell activity in a transplant recipient is inhibited by administration of an agent which inhibits T cell activity. The agent which inhibits T cell activity can be, for example, a drug which inhibits T cell activity or an antibody which depletes T cells or inhibits T cell proliferation.

Preferably, the agent which inhibits T cell activity is an immunosuppressive drug, such as cyclosporin A. Other immunosuppressive drugs which can be used include FK506 and RS-61443. Such immunosuppressive drugs can be used in conjunction with a steroid (e.g., a glucocorticoid such as prednisone, methylprednisolone and dexamethasone) or a chemotherapeutic agent (e.g., azathioprine and cyclophosphamide), or both. The immunosuppressive drug can be administered to the subject transiently, for a period of time sufficient to induce tolerance to the transplanted cells. For example, the drug can be administered to the subject for not less than three days and not more than three months, preferably, not less than one week and not more than one month, following transplantation. An immunosuppressive drug is administered at a dosage sufficient to achieve the desired therapeutic effect. Dosages of an immunosuppressive drug preferably are adjusted to maintain serum levels in a subject between about 100-200 ng/ml. A typical dosage range for cyclosporin A is between about 1 and 30 mg/kg of body weight per day.

Other agents which inhibit T cell activity which can be used in the method of the invention include antibodies which can deplete or sequester T cells *in vivo*. Suitable antibodies include antibodies which bind to antigens on the surface of T cells, such as CD2, CD3, CD4 or CD8. Alternatively, an antibody which binds a T cell growth factor or growth factor receptor (e.g., IL-2 or the IL-2 receptor) can be used to inhibit T cell proliferation in the subject.

In addition to inhibiting rejection of transplanted cells, the methods of the invention have the advantage of inducing donor cell-specific tolerance to the transplanted cells in a transplant recipient. The invention thus provides methods for successful transplantation of cells into an allogeneic or xenogeneic transplant recipient which avoids life-long generalized immunosuppression of the subject.

Brief Description of the Drawings

Figure 1 shows a graph depicting the percent of serum cholesterol over time of WHHL rabbits transplanted with either: 1) porcine hepatocytes double masked with F(ab')₂ fragments of the monoclonal antibody PT-85 and F(ab')₂ fragments of the monoclonal antibody 9-3; 2) porcine hepatocytes masked with F(ab')₂ fragments of the monoclonal antibody PT-85; or 3) unmasked porcine hepatocytes, and subject to short term (two weeks) cyclosporin A treatment.

Figure 2 shows a graph depicting the percent of serum cholesterol over time of WHHL rabbits transplanted with either: 1) porcine hepatocytes masked with F(ab')₂ fragments of the monoclonal antibody PT-85; or 2) unmasked hepatocytes, and subject to single dose cyclosporin A treatment.

Figure 3 shows a graph depicting the percent of serum cholesterol over time of WHHL rabbits transplanted with either 1) porcine hepatocytes masked with F(ab')₂ fragments

of the monoclonal antibody PT-85 but not subject to cyclosporin A treatment; 2) unmasked porcine hepatocytes but not subject to cyclosporin A treatment; or 3) porcine hepatocytes masked with F(ab')₂ fragments of the monoclonal antibody PT-85 and subject to single dose cyclosporin A treatment.

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Detailed Description of the Invention

The invention features improved methods for transplanting cells into an allogeneic or xenogeneic recipient such that rejection of transplanted cells by the recipient is inhibited and tolerance to the transplanted cells is induced in the recipient. The methods involve administration to a recipient subject of two compositions: the cells to be transplanted; and an agent which inhibits T cell activity in the subject. The cells which are administered to the subject have been treated prior to transplantation such that rejection of the cells by the subject is inhibited. The cells to be transplanted into a recipient are treated such that at least one antigen on the surface of the cell is altered prior to transplantation to modify an interaction between the antigen and a hematopoietic cell (e.g., T lymphocyte) in the recipient, thereby inhibiting rejection of the cells by the recipient. In addition to administration of the modified cell, the recipient is treated with an agent which inhibits T cell activity in the recipient to further inhibit rejection of the transplanted cells. It has been found that the use of these two treatments in combination is more effective for successful transplantation of allogeneic or xenogeneic cells than use of either method alone.

As used herein, the term "subject" is intended to include living organisms in which an immune response is elicited against allogeneic or xenogeneic cells, e.g., mammals, preferably humans. A "recipient subject" is a subject into which cells have been transplanted or are to be transplanted. A recipient subject may be allogeneic to the transplanted cells (i.e., of the same species) or may be xenogeneic to the transplanted cells (i.e., of a different species). The temporal relationship between administration of the cell and administration of the agent depends in part upon the nature of the agent used to inhibit T cell activity. Typically, the two compositions are administered contemporaneously, e.g. within several days of each other. Preferably, the cell and the agent are administered to the subject simultaneously or the agent is administered to the subject prior to administration of the cell.

The two aspects of the method of the invention for inhibiting the rejection of transplanted cells are described in further detail in the following subsections.

I. Alteration of an Antigen on a Cell to be Transplanted

One aspect of the method of the invention involves alteration of an antigen on the surface of the cell to be transplanted prior to transplantation to inhibit rejection of the cell following transplantation. In an unaltered state, the antigen on the cell surface stimulates an immune response against the cell (also referred to herein as the donor cell) when the cell is administered to a subject (also referred to herein as the recipient or host). By altering the

antigen, the normal immunological recognition of the donor cell by the immune system cells of the recipient is disrupted and additionally, "abnormal" immunological recognition of this altered form of the antigen can lead to donor cell-specific long term unresponsiveness in the recipient. Thus, alteration of an antigen on the donor cell prior to administering the cell to a recipient interferes with the initial phase of recognition of the donor cell by the cells of the host's immune system subsequent to administration of the cell. Furthermore, alteration of the antigen can induce immunological nonresponsiveness or tolerance, thereby preventing the induction of the effector phases of an immune response (e.g., cytotoxic T cell generation, antibody production etc.) which are ultimately responsible for rejection of foreign cells in a normal immune response. As used herein, the term "altered" encompasses changes that are made to a donor cell antigen which reduce the immunogenicity of the antigen to thereby interfere with immunological recognition of the antigen by the recipient's immune system. Preferably immunological nonresponsiveness to the donor cells in the recipient subject is generated as a result of alteration of the antigen. The term "altered" is not intended to include complete elimination of the antigen on the donor cell since delivery of an inappropriate or insufficient signal to the host's immune cells (e.g., T lymphocytes) may be necessary to achieve immunological nonresponsiveness.

Antigens to be altered according to the current invention include antigens on a donor cell which can interact with a hematopoietic cell in an allogeneic or xenogeneic recipient and thereby stimulate a specific immune response against the donor cell in the recipient. The interaction between the antigen and the hematopoietic cell may be an indirect interaction (e.g., mediated by soluble factors which induce a response in the hematopoietic cell, e.g., humoral mediated) or, preferably, is a direct interaction between the antigen and a molecule present on the surface of the hematopoietic cell (i.e., cell-cell mediated). As used herein, the phrase "hematopoietic cell" is intended to include T lymphocytes, B lymphocytes, monocytes and other antigen presenting cells. In a preferred embodiment, the antigen is one which interacts with a T lymphocyte in the recipient (e.g., the antigen normally binds to a receptor on the surface of a T lymphocyte).

In a preferred embodiment, the antigen on the donor cell to be altered is an MHC class I antigen. MHC class I antigens are present on almost all cell types. In a normal immune response, self MHC molecules function to present antigenic peptides to a T cell receptor (TCR) on the surface of self T lymphocytes. In immune recognition of allogeneic or xenogeneic cells, foreign MHC antigens (most likely together with a peptide bound thereto) on donor cells are recognized by the T cell receptor on host T cells to elicit an immune response. MHC class I antigens on a donor cell are altered to interfere with their recognition by T cells in an allogeneic or xenogeneic host (e.g., a portion of the MHC class I antigen which is normally recognized by the T cell receptor is blocked or "masked" such that normal recognition of the MHC class I antigen can no longer occur). Additionally, an altered form of an MHC class I antigen which is exposed to host T cells (i.e., available for presentation to the

host T cell receptor) may deliver an inappropriate or insufficient signal to the host T cell such that, rather than stimulating an immune response against the allogeneic or xenogeneic cell, donor cell-specific T cell non-responsiveness is induced. For example, it is known that T cells which receive an inappropriate or insufficient signal through their T cell receptor (e.g., by binding to an MHC antigen in the absence of a costimulatory signal, such as that provided by B7) become anergic rather than activated and can remain refractory to restimulation for long periods of time (see for example Damle et al. (1981) *Proc. Natl. Acad. Sci. USA* 78:5096-5100; Lesslauer et al. (1986) *Eur. J. Immunol.* 16:1289-1295; Gimmi, et al. (1991) *Proc. Natl. Acad. Sci. USA* 88: 6575-6579; Linsley et al. (1991) *J. Exp. Med.* 173:721-730; Koulova et al. (1991) *J. Exp. Med.* 173:759-762; Razi-Wolf, et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:4210-4214).

Alternative to MHC class I antigens, the antigen to be altered on a donor cell can be an MHC class II antigen. Similar to MHC class I antigens, MHC class II antigens function to present antigenic peptides to a T cell receptor on T lymphocytes. However, MHC class II antigens are present on a limited number of cell types (primarily B cells, macrophages, dendritic cells, Langerhans cells and thymic epithelial cells). In addition to or alternative to MHC antigens, other antigens on a donor cell which interact with molecules on host T cells and which are known to be involved in immunological rejection of allogeneic or xenogeneic cells can be altered. Other donor cell antigens known to interact with host T cells and contribute to rejection of a donor cell include molecules which function to increase the avidity of the interaction between a donor cell and a host T cell. Due to this property, these molecules are typically referred to as adhesion molecules (although they may serve other functions in addition to increasing the adhesion between a donor cell and a host T cell). Examples of preferred adhesion molecules which can be altered according to the invention include LFA-3 and ICAM-1. These molecules are ligands for the CD2 and LFA-1 receptors, respectively, on T cells. By altering an adhesion molecule on the donor cell, (such as LFA-3, ICAM-1 or a similarly functioning molecule), the ability of the host's T cells to bind to and interact with the donor cell is reduced. Both LFA-3 and ICAM-1 are found on endothelial cells found within blood vessels in transplanted organs such as kidney and heart. Altering these antigens can facilitate transplantation of any vascularized implant, by altering recognition of those antigens by CD2+ and LFA-1+ host T-lymphocytes.

The presence of MHC molecules or adhesion molecules such as LFA-3, ICAM-1 etc. on a particular donor cell can be assessed by standard procedures known in the art. For example, the donor cell can be reacted with a labeled antibody directed against the molecule to be detected (e.g., MHC molecule, ICAM-1, LFA-1 etc.) and the association of the labeled antibody with the cell can be measured by a suitable technique (e.g., immunohistochemistry, flow cytometry etc.).

A preferred method for altering an antigen on a donor cell to inhibit an immune response against the cell is to contact the cell with a molecule which binds to the antigen on

the cell surface. It is preferred that the cell be contacted with the molecule which binds to the antigen prior to administering the cell to a recipient (i.e., the cell is contacted with the molecule *in vitro*). For example, the cell can be incubated with the molecule which binds the antigen under conditions which allow binding of the molecule to the antigen and then any unbound molecule can be removed (such as described in the Examples below). Following administration of the modified cell to a recipient, the molecule remains bound to the antigen on the cell for a sufficient time to interfere with immunological recognition by host cells and induce non-responsiveness in the recipient.

Preferably, the molecule for binding to an antigen on a donor cell is an antibody, or fragment or derivative thereof which retains the ability to bind to the antigen. For use in therapeutic applications, it is necessary that the antibody which binds the antigen to be altered be unable to fix complement, thus preventing donor cell lysis. Antibody complement fixation can be prevented by deletion of an Fc portion of an antibody, by using an antibody isotype which is not capable of fixing complement, or, less preferably, by using a complement fixing antibody in conjunction with a drug which inhibits complement fixation. Alternatively, amino acid residues within the Fc region which are necessary for activating complement (see e.g., Tan et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:162-166; Duncan and Winter (1988) *Nature* 332: 738-740) can be mutated to reduce or eliminate the complement-activating ability of an intact antibody. Likewise, amino acids residues within the Fc region which are necessary for binding of the Fc region to Fc receptors (see e.g., Canfield, S.M. and S.L. Morrison (1991) *J. Exp. Med.* 173:1483-1491; and Lund, J. et al. (1991) *J. Immunol.* 147:2657-2662) can also be mutated to reduce or eliminate Fc receptor binding if an intact antibody is to be used.

A preferred antibody fragment for altering an antigen is an F(ab')₂ fragment. Antibodies can be fragmented using conventional techniques. For example, the Fc portion of an antibody can be removed by treating an intact antibody with pepsin, thereby generating an F(ab')₂ fragment. In a standard procedure for generating F(ab')₂ fragments, intact antibodies are incubated with immobilized pepsin and the digested antibody mixture is applied to an immobilized protein A column. The free Fc portion binds to the column while the F(ab')₂ fragments pass through the column. The F(ab')₂ fragments can be further purified by HPLC or FPLC. F(ab')₂ fragments can be treated to reduce disulfide bridges to produce Fab' fragments.

An antibody, or fragment or derivative thereof, to be used to alter an antigen can be derived from polyclonal antisera containing antibodies reactive with a number of epitopes on an antigen. Preferably, the antibody is a monoclonal antibody directed against the antigen. Polyclonal and monoclonal antibodies can be prepared by standard techniques known in the art. For example, a mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with the antigen or with a cell which expresses the antigen (e.g., on the cell surface) to elicit an antibody response against the antigen in the mammal. Alternatively, tissue or a whole organ

which expresses the antigen can be used to elicit antibodies. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay can be used with the antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera. To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art. For example, the hybridoma technique originally developed by Kohler and Milstein ((1975) *Nature* 256:495-497) as well as other techniques such as the human B-cell hybridoma technique (Kozbar et al., (1983) *Immunol. Today* 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al. (1985) *Monoclonal Antibodies in Cancer Therapy*, Allen R. Bliss, Inc., pages 77-96) can be used. Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the antigen and monoclonal antibodies isolated.

Another method of generating specific antibodies, or antibody fragments, reactive against the antigen is to screen expression libraries encoding immunoglobulin genes, or portions thereof, expressed in bacteria with the antigen (or a portion thereof). For example, complete Fab fragments, V_H regions, F_V regions and single chain antibodies can be expressed in bacteria using phage expression libraries. See for example Ward et al., (1989) *Nature* 341:544-546; Huse et al., (1989) *Science* 246:1275-1281; and McCafferty et al. (1990) *Nature* 348:552-554. Alternatively, a SCID-hu mouse can be used to produce antibodies, or fragments thereof (available from Genpharm). Antibodies of the appropriate binding specificity which are made by these techniques can be used to alter an antigen on a donor cell.

An antibody, or fragment thereof, produced in a non-human subject can be recognized to varying degrees as foreign when the antibody is administered to a human subject (e.g., when a donor cell with an antibody bound thereto is administered to a human subject) and an immune response against the antibody may be generated in the subject. One approach for minimizing or eliminating this problem is to produce chimeric or humanized antibody derivatives, i.e., antibody molecules comprising portions which are derived from non-human antibodies and portions which are derived from human antibodies. Chimeric antibody molecules can include, for example, an antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. A variety of approaches for making chimeric antibodies have been described. See, for example, Morrison et al., *Proc. Natl. Acad. Sci. U.S.A.* 81, 6851 (1985); Takeda et al., *Nature* 314, 452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom Patent GB 2177096B. For use in therapeutic applications, it is preferred that an antibody used to alter a donor cell antigen not contain an Fc portion. Thus, a humanized $F(ab')_2$ fragment in which

parts of the variable region of the antibody, especially the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin is a preferred antibody derivative. Such altered immunoglobulin molecules may be made by any of several techniques known in the art, (e.g., Teng et al., *Proc. Natl. Acad. Sci. U.S.A.*, 80, 7308-7312 (1983); Kozbor et al., *Immunology Today*, 4, 7279 (1983); Olsson et al., *Meth. Enzymol.*, 92, 3-16 (1982)), and are preferably made according to the teachings of PCT Publication WO92/06193 or EP 0239400. Humanized antibodies can be commercially produced by, for example, Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.

Each of the cell surface antigens to be altered, e.g., MHC class I antigens, MHC class II antigens, LFA-3 and ICAM-1 are well-characterized molecules and antibodies to these antigens are commercially available. For example, an antibody directed against human MHC class I antigens (i.e., an anti-HLA class I antibody), W6/32, is available from the American Type Culture Collection (ATCC HB 95). This antibody was raised against human tonsillar lymphocyte membranes and binds to HLA-A, HLA-B and HLA-C (Barnstable, C.J. et al. (1978) *Cell* 14:9-20). Another anti-MHC class I antibody which can be used is PT85 (see Davis, W.C. et al. (1984) *Hybridoma Technology in Agricultural and Veterinary Research*. N.J. Stern and H.R. Gamble, eds., Rowman and Allenheld Publishers, Totowa, NJ, p121; commercially available from Veterinary Medicine Research Development, Pullman, WA). This antibody was raised against swine leukocyte antigens (SLA) and binds to class I antigens from several different species (e.g., pig, human, mouse, goat). An anti-ICAM-1 antibody can be obtained from AMAC, Inc., Maine. Hybridoma cells producing anti-LFA-3 can be obtained from the American Type Culture Collection, Rockville, Maryland.

A suitable antibody, or fragment or derivative thereof, for use in the invention can be identified based upon its ability to inhibit the immunological rejection of allogeneic or xenogeneic cells using a protocol such as that described in the Examples. Briefly, the antibody (or antibody fragment) is incubated for a short period of time (e.g., 30 minutes at room temperature) with cells or tissue to be transplanted and any unbound antibody is washed away. The cells or tissue are then transplanted into a recipient animal. The ability of the antibody pretreatment to inhibit or prevent rejection of the transplanted cells or tissue is then determined by monitoring for rejection of the cells or tissue compared to untreated controls.

It is preferred that an antibody, or fragment or derivative thereof, which is used to alter an antigen have an affinity for binding to the antigen of at least 10^{-7} M. The affinity of an antibody or other molecule for binding to an antigen can be determined by conventional techniques (see Masan, D.W. and Williams, A.F. (1980) *Biochem. J.* 187:1-10). Briefly, the antibody to be tested is labeled with 125 I and incubated with cells expressing the antigen at increasing concentrations until equilibrium is reached. Data are plotted graphically as [bound antibody]/[free antibody] versus [bound antibody] and the slope of the line is equal to the K_D (Scatchard analysis).

Other molecules which bind to an antigen on a donor cell and produce a functionally similar result as antibodies, or fragments or derivatives thereof, (e.g., other molecules which interfere with the interaction of the antigen with a hematopoietic cell and induce immunological nonresponsiveness) can be used to alter the antigen on the donor cell. One such molecule is a soluble form of a ligand for an antigen (e.g., a receptor) on the donor cell which could be used to alter the antigen on the donor cell. For example, a soluble form of CD2 (i.e., comprising the extracellular domain of CD2 without the transmembrane or cytoplasmic domain) can be used to alter LFA-3 on the donor cell by binding to LFA-3 on donor cells in a manner analogous to an antibody. Alternatively, a soluble form of LFA-1 can be used to alter ICAM-1 on the donor cell. A soluble form of a ligand can be made by standard recombinant DNA procedures, using a recombinant expression vector containing DNA encoding the ligand encompassing an extracellular domain (i.e., lacking DNA encoding the transmembrane and cytoplasmic domains). The recombinant expression vector encoding the extracellular domain of the ligand can be introduced into host cells to produce a soluble ligand, which can then be isolated. Soluble ligands of use have a binding affinity for the receptor on the donor cell sufficient to remain bound to the receptor to interfere with immunological recognition and induce non-responsiveness when the cell is administered to a recipient (e.g., preferably, the affinity for binding of the soluble ligand to the receptor is at least about 10^{-7} M). Additionally, the soluble ligand can be in the form of a fusion protein comprising the receptor binding portion of the ligand fused to another protein or portion of a protein. For example, an immunoglobulin fusion protein which includes an extracellular domain, or functional portion of CD2 or LFA-1 linked to an immunoglobulin heavy chain constant region (e.g., the hinge, CH2 and CH3 regions of a human immunoglobulin such as IgG1) can be used. Immunoglobulin fusion proteins can be prepared, for example, according to the teachings of Capon, D.J. et al. (1989) *Nature* 337:525-531 and U.S. Patent No. 5,116,964 to Capon and Lasky.

Another type of molecule which can be used to alter an MHC antigen (e.g., and MHC class I antigen) is a peptide which binds to the MHC antigen and interferes with the interaction of the MHC antigen with a T lymphocyte. In one embodiment, the soluble peptide mimics a region of the T cell receptor which contacts the MHC antigen. This peptide can be used to interfere with the interaction of the intact T cell receptor (on a T lymphocyte) with the MHC antigen. Such a peptide binds to a region of the MHC molecule which is specifically recognized by a portion of the T cell receptor (e.g., the alpha-1 or alpha-2 loop of an MHC class I antigen), thereby altering the MHC class I antigen and inhibiting recognition of the antigen by the T cell receptor. In another embodiment, the soluble peptide mimics a region of a T cell surface molecule which contacts the MHC antigen, such as a region of the CD8 molecule which contacts an MHC class I antigen or a region of a CD4 molecule which contacts an MHC class II antigen. For example, a peptide which binds to a region of the alpha-3 loop of an MHC class I antigen can be used to inhibit binding to CD8 to the antigen,

thereby inhibiting recognition of the antigen by T cells. T cell receptor-derived peptides have been used to inhibit MHC class I-restricted immune responses (see e.g., Clayberger, C. et al. (1993) *Transplant Proc.* 25:477-478) and prolong allogeneic skin graft survival *in vivo* when injected subcutaneously into the recipient (see e.g., Goss, J.A. et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:9872-9876).

An antigen on a donor cell further can be altered by using two or more molecules which bind to the same or different antigen. For example, two different antibodies with specificity for two different epitopes on the same antigen can be used (e.g., two different anti-MHC class I antibodies can be used in combination). Alternatively, two different types of molecules which bind to the same antigen can be used (e.g., an anti-MHC class I antibody and an MHC class I-binding peptide). A preferred combination of anti-MHC class I antibodies which can be used with human cells is the W6/32 antibody and the PT85 antibody or F(ab')₂ fragments thereof. When the donor cell to be administered to a subject bears more than one hematopoietic cell-interactive antigen, two or more treatments can be used together. For example, two antibodies, each directed against a different antigen (eg., an anti-MHC class I antibody and an anti-ICAM-1 antibody) can be used in combination or two different types of molecules, each binding to a different antigen, can be used (e.g., an anti-ICAM-1 antibody and an MHC class I-binding peptide). Alternatively, polyclonal antisera generated against the entire donor cell or tissue containing donor cells can be used, following removal of the Fc region, to alter multiple cell surface antigens of the donor cells.

The ability of two different monoclonal antibodies which bind to the same antigen to bind to different epitopes on the antigen can be determined using a competition binding assay. Briefly, one monoclonal antibody is labeled and used to stain cells which express the antigen. The ability of the unlabeled second monoclonal antibody to inhibit the binding of the first labeled monoclonal antibody to the antigen on the cells is then assessed. If the second monoclonal antibody binds to a different epitope on the antigen than does the first antibody, the second antibody will be unable to competitively inhibit the binding of the first antibody to the antigen.

A preferred method for altering at least two different epitopes on an antigen on a donor cell to inhibit an immune response against the cell is to contact the cell with at least two different molecules which bind to the epitopes. It is preferred that the cell be contacted with at least two different molecules which bind to the different epitopes prior to administering the cell to a recipient (i.e., the cell is contacted with the molecule *in vitro*). For example, the cell can be incubated with the molecules which bind to the epitopes under conditions which allow binding of the molecules to the epitopes and then any unbound molecules can be removed. Following administration of the donor cell to a recipient, the molecules remain bound to the epitopes on the surface antigen for a sufficient time to interfere with immunological recognition by host cells and induce non-responsiveness in the recipient.

Alternative to binding a molecule (e.g., an antibody) to an antigen on a donor cell to inhibit immunological rejection of the cell, the antigen on the donor cell can be altered by other means. For example, the antigen can be directly altered (e.g., mutated) such that it can no longer interact normally with a hematopoietic cell (e.g., a T lymphocyte) in an allogeneic or xenogeneic recipient and induces immunological non-responsiveness to the donor cell in the recipient. For example, a mutated form of a class I MHC antigen or adhesion molecule (e.g., LFA-3 or ICAM-1) which does not contribute to T cell activation but rather delivers an inappropriate or insufficient signal to a T cell upon binding to a receptor on the T cell can be created by mutagenesis and selection. A nucleic acid encoding the mutated form of the antigen can then be inserted into the genome of a non-human animal, either as a transgene or by homologous recombination (to replace the endogenous gene encoding the wild-type antigen). Cells from the non-human animal which express the mutated form of the antigen can then be used as donor cells for transplantation into an allogeneic or xenogeneic recipient.

Alternatively, an antigen on the donor cell can be altered by downmodulating or altering its level of expression on the surface of the donor cell such that the interaction between the antigen and a recipient hematopoietic cell is modified. By decreasing the level of surface expression of one or more antigens on the donor cell, the avidity of the interaction between the donor cell and the hematopoietic cell (e.g., T lymphocyte) is reduced. The level of surface expression of an antigen on the donor cell can be down-modulated by inhibiting the transcription, translation or transport of the antigen to the cell surface. Agents which decrease surface expression of the antigen can be contacted with the donor cell. For example, a number of oncogenic viruses have been demonstrated to decrease MHC class I expression in infected cells (see e.g., Travers et al. (1980) *Int'l. Symp. on Aging in Cancer*, 175180; Rees et al. (1988) *Br. J. Cancer*, 57:374-377). In addition, it has been found that this effect on MHC class I expression can be achieved using fragments of viral genomes, in addition to intact virus. For example, transfection of cultured kidney cells with fragments of adenovirus causes elimination of surface MHC class I antigenic expression (Whoshi et al. (1988) *J. Exp. Med.* 168:2153-2164). For purposes of decreasing MHC class I expression on the surfaces of donor cells, viral fragments which are non-infectious are preferable to whole viruses.

Alternatively, the level of an antigen on the donor cell surface can be altered by capping the antigen. Capping is a term referring to the use of antibodies to cause aggregation and inactivation of surface antigens. To induce capping, a tissue is contacted with a first antibody specific for an antigen to be altered, to allow formation of antigen-antibody immune complexes. Subsequently, the tissue is contacted with a second antibody which forms immune complexes with the first antibody. As a result of treatment with the second antibody, the first antibody is aggregated to form a cap at a single location on the cell surface. The technique of capping is well known and has been described, e.g., in Taylor et al. (1971), *Nat. New Biol.* 233:225-227; and Santiso et al. (1986), *Blood*, 67:343-349. To alter MHC class I antigens, donor cells are incubated with a first antibody (e.g., W6/32 antibody, PT85

antibody) reactive with MHC class I molecules, followed by incubation with a second antibody reactive with the donor species, e.g., goat anti-mouse antibody, to result in aggregation.

5 II. Administration of an Agent which Inhibits T Cell Activity

To inhibit rejection of transplanted cells and to achieve immunological non-responsiveness in an allogeneic or xenogeneic transplant recipient, the method of the invention requires administration to the recipient of an agent which inhibits T cell activity. As used herein, an agent which inhibits T cell activity is defined as an agent which results in
10 removal (e.g., sequestration) or destruction of T cells within a subject or inhibits T cell functions within the subject (i.e., T cells may still be present in the subject but are in a non-functional state, such that they are unable to proliferate or elicit or perform effector functions, e.g., cytokine production, cytotoxicity etc.). The term "T cell" encompasses mature
15 peripheral blood T cells lymphocytes. The agent which inhibits T cell activity may also inhibit the activity or maturation of immature T cells (e.g., thymocytes).

A preferred agent for use in inhibiting T cell activity in a recipient subject is an immunosuppressive drug. The term "immunosuppressive drug" is intended to include pharmaceutical agents which inhibit or interfere with normal immune function. A preferred
20 immunosuppressive drug is cyclosporin A. Other immunosuppressive drugs which can be used include FK506 and RS-61443. In one embodiment, the immunosuppressive drug is administered in conjunction with at least one other therapeutic agent. Additional therapeutic
25 agents which can be administered include steroids (e.g., glucocorticoids such as prednisone, methyl prednisolone and dexamethasone) and chemotherapeutic agents (e.g., azathioprine and cyclophosphamide). In another embodiment, an immunosuppressive drug is
30 administered in conjunction with both a steroid and a chemotherapeutic agent. Suitable immunosuppressive drugs are commercially available (e.g., cyclosporin A is available from Sandoz, Corp., East Hanover, NJ).

An immunosuppressive drug is administered in a formulation which is compatible with the route of administration. Suitable routes of administration include intravenous
35 injection (either as a single infusion, multiple infusions or as an intravenous drip over time), intraperitoneal injection, intramuscular injection and oral administration. For intravenous injection, the drug can be dissolved in a physiologically acceptable carrier or diluent (e.g., a buffered saline solution) which is sterile and allows for syringability. Dispersions of drugs can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Convenient routes of administration and carriers for immunosuppressive drugs are known in
the art. For example, cyclosporin A can be administered intravenously in a saline solution, or orally, intraperitoneally or intramuscularly in olive oil or other suitable carrier or diluent.

An immunosuppressive drug is administered to a recipient subject at a dosage sufficient to achieve the desired therapeutic effect (e.g., inhibition of rejection of transplanted

cells). Dosage ranges for immunosuppressive drugs, and other agents which can be coadministered therewith (e.g., steroids and chemotherapeutic agents), are known in the art (see e.g., Freed et al. *New Engl. J. Med.* (1992) 327:1549; Spencer et al. (1992) *New Engl. J. Med.* 327:1541; Widner et al. (1992) *New Engl. J. Med.* 327:1556; Lindvall et al. (1992) *Ann. Neurol.* 31:155; and Lindvall et al. (1992) *Arch. Neurol.* 46:615). A preferred dosage range for immunosuppressive drugs, suitable for treatment of humans, is about 1-30 mg/kg of body weight per day. A preferred dosage range for cyclosporin A is about 1-10 mg/kg of body weight per day, more preferably about 1-5 mg/kg of body weight per day. Dosages can be adjusted to maintain an optimal level of the immunosuppressive drug in the serum of the recipient subject. For example, dosages can be adjusted to maintain a preferred serum level for cyclosporin A in a human subject of about 100-200 ng/ml. It is to be noted that dosage values may vary according to factors such as the disease state, age, sex, and weight of the individual. Dosage regimens may be adjusted over time to provide the optimum therapeutic response according to the individual need and the professional judgment of the person administering or supervising the administration of the compositions, and that the dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

In one embodiment of the invention, an immunosuppressive drug is administered to a subject transiently for a sufficient time to induce tolerance to the transplanted cells in the subject. Transient administration of an immunosuppressive drug has been found to induce long-term graft-specific tolerance in a graft recipient (see Examples below and Brunson et al. (1991) *Transplantation* 52:545; Hutchinson et al. (1981) *Transplantation* 32:210; Green et al. (1979) *Lancet* 2:123; Hall et al. (1985) *J. Exp. Med.* 162:1683). Administration of the drug to the subject can begin prior to transplantation of the cells into the subject. For example, initiation of drug administration can be a few days (e.g., one to three days) before transplantation. Alternatively, drug administration can begin the day of transplantation or a few days (generally not more than three days) after transplantation. Administration of the drug is continued for sufficient time to induce donor cell-specific tolerance in the recipient such that donor cells will continue to be accepted by the recipient when drug administration ceases. For example, the drug can be administered for as short as three days or as long as three months following transplantation. Typically, the drug is administered for at least one week but not more than one month following transplantation. Induction of tolerance to the transplanted cells in a subject is indicated by the continued acceptance of the transplanted cells after administration of the immunosuppressive drug has ceased. Acceptance of transplanted tissue can be determined morphologically (e.g., with skin grafts by examining the transplanted tissue or by biopsy) or by assessment of the functional activity of the graft. For example, acceptance of pancreatic islet cells can be determined by measuring insulin production, acceptance of liver cells can be determined by assessing liver function or acceptance of neural cells can be determined by assessing neural cell function.

Another type of agent which can be used to inhibit T cell activity in a subject is an antibody, or fragment or derivative thereof, which depletes or sequesters T cells in a recipient. Antibodies which are capable of depleting or sequestering T cells *in vivo* when administered to a subject are known in the art. Typically, these antibodies bind to an antigen on the surface of a T cell. Polyclonal antisera can be used, for example anti-lymphocyte serum. Alternatively, one or more monoclonal antibodies can be used. Preferred T cell-depleting antibodies include monoclonal antibodies which bind to CD2, CD3, CD4 or CD8 on the surface of T cells. Antibodies which bind to these antigens are known in the art and are available (e.g., from American Type Culture Collection). A preferred monoclonal antibody for binding to CD3 on human T cells is OKT3 (ATCC CRL 8001). The binding of an antibody to surface antigens on a T cell can facilitate sequestration of T cells in a subject and/or destruction of T cells in a subject by endogenous mechanisms. Alternatively, a T cell-depleting antibody which binds to an antigen on a T cell surface can be conjugated to a toxin (e.g., ricin) or other cytotoxic molecule (e.g., a radioactive isotope) to facilitate destruction of T cells upon binding of the antibody to the T cells.

Another type of antibody which can be used to inhibit T cell activity in a recipient subject is an antibody which inhibits T cell proliferation. For example, an antibody directed against a T cell growth factor, such as IL-2, or a T cell growth factor receptor, such as the IL-2 receptor, can inhibit proliferation of T cells (see e.g., DeSilva, D.R. et al. (1991) *J. Immunol.* 147:3261-3267). Accordingly, an anti-IL-2 or an anti-IL-2 receptor antibody can be administered to a recipient to inhibit rejection of a transplanted cell (see e.g. Wood et al. (1992) *Neuroscience* 49:410). Additionally, both an anti-IL-2 and an anti-IL-2 receptor antibody can be coadministered to inhibit T cell activity or can be administered with another antibody (e.g., which binds to a surface antigen on T cells).

An antibody which depletes, sequesters or inhibits T cells within a recipient can be administered at a dose and for an appropriate time to inhibit rejection of cells upon transplantation. Antibodies are preferably administered intravenously in a pharmaceutically acceptable carrier or diluent (e.g., a sterile saline solution). Antibody administration can begin prior to transplantation (e.g., one to five days prior to transplantation) and can continue on a daily basis after transplantation to achieve the desired effect (e.g., up to fourteen days after transplantation). A preferred dosage range for administration of an antibody to a human subject is about 0.1-0.3 mg/kg of body weight per day. Alternatively, a single high dose of antibody (e.g., a bolus at a dosage of about 10 mg/kg of body weight) can be administered to a human subject on the day of transplantation. The effectiveness of antibody treatment in depleting T cells from the peripheral blood can be determined by comparing T cell counts in blood samples taken from the subject before and after antibody treatment. Dosage regimens can be adjusted over time to provide the optimum therapeutic response according to the individual need and the professional judgment of the person administering or supervising the

administration of the compositions. Dosage ranges set forth herein are exemplary only and are not intended to limit the scope or practice of the claimed composition.

III. Uses of the Method of the Invention

5 Cells having at least one surface antigen altered according to the invention can be administered to a subject (i.e., transplanted into the subject) for therapeutic purposes. The term "subject" is intended to include mammals in which an immune response is elicited against allogeneic or xenogeneic cells. Examples of subjects include humans, monkeys, pigs, 10 dogs, cats, mice, rats, and transgenic species thereof. A "recipient subject" as used herein is a subject into which foreign cells are transplanted or are to be transplanted. A cell can be administered to a subject by any appropriate route which results in delivery of cell to a desired location in the subject. For example, cells can be administered intravenously, subcutaneously, intramuscularly, intracerebrally, subcapsularly (e.g., under the kidney capsule) or intraperitoneally. Cells can be administered in a physiologically compatible 15 carrier, such as a buffered saline solution. When cells are within a tissue or organ, the tissue or organ can be transplanted into a suitable location in the subject by conventional techniques to administer the cells to the subject.

The methods of the invention can be applied to any type of cell which is suitable for transplantation (i.e., any type of cell which can be isolated or obtained in a form that can be 20 transplanted to another subject). The cells can be human cells or non-human cells. Preferred non-human cells are porcine cells. Preferred cell types for use in the method of the invention are cells which can provide a therapeutic function in a disease or disorder. Examples of such cells include muscle cells (e.g., myoblasts, myocytes, myotubes), liver cells, pancreatic islet 25 cells, neural cells and hematopoietic cells. For example, muscle cells can be transplanted into subjects suffering from a muscular dystrophy (e.g., Duchenne muscular dystrophy), pancreatic islet cells can be transplanted into a subject suffering from diabetes, neural cells can be transplanted into a subject suffering from Parkinson's disease or Huntington's disease, liver cells can be transplanted into a subject with hepatic cell dysfunction (e.g. in 30 hypercholesterolemia, hemophilia B or inherited emphysema), and hematopoietic cells can be transplanted into patients with hematopoietic or immunological dysfunction. Liver tissue can be obtained, for example, from brain dead donors or from non-human animals such as pigs. The cells can be dissociated by digestion with collagenase. Viable cells can be obtained and washed by centrifugation (at 700 x g), elution, and resuspension. At least one antigen on the surface of the liver cells (e.g., MHC class I antigen) is altered as described herein. Following 35 alteration of the antigen(s), cells are administered through the portal vein to the liver of the recipient patient. In another embodiment, nerve cells obtained from a source (such as an abortus) are treated to alter a surface antigen and stereotaxically localized into the desired area of the brain, such as the corpus striatum. Dopaminergic or GABA-ergic neurons are used for the treatment of Parkinson's or Huntington's disease, respectively. In another

embodiment, muscle cells can be obtained from a donor (e.g., by biopsy of a living related donor or from a brain dead donor) using a 14-16 gauge cutting trochar into a 1-2 inch skin incision. The fresh muscle plug can be lightly digested to a single cell suspension using collagenase, trypsin and dispase at 37° C. Floating debris is removed with a pipette and media washes and the viable cell pellet is counted after centrifugation at 1000 rpm for 10 minutes. The cell count is then used to calculate the amount of antibody fragments (or other suitable molecule, e.g. peptide) to be used to alter a surface antigen on the muscle cells. Muscle cells are injected intramuscularly into a recipient patient in need of an increased store of muscle, e.g., an elderly patient with severe muscle wasting, or injected into a muscle group of a patient afflicted with Becker's or Duchenne muscular dystrophy.

Recipient subjects are further treated with a T cell inhibitory agent according to the invention. Treatment can begin prior to, concurrent with or following transplantation of cells. The combination therapy taught by the invention provides a therapeutic regimen for transplantation of allogeneic or xenogeneic cells into a recipient subject which is more effective than either alteration of donor cell surface antigens or treatment of the recipient with a T cell inhibitory agent alone.

This invention is further illustrated by the following Examples which should not be construed as limiting. The contents of all references and published patents and patent applications cited throughout the application are hereby incorporated by reference.

EXAMPLE I: IMPLANTATION OF FETAL PORCINE STRIATAL CELLS IN A RAT MODEL OF HUNTINGTON'S DISEASE

5 Excitotoxic lesions of the striatum in rats provide an animal model of the neuropathology seen in Huntington's disease. In order to repair the circuitry of the striatum, fetal porcine striatal cells were transplanted into the damaged region. The lateral ganglionic eminence (the structure that later develops into adult striatum) of the porcine fetal brain was micro Surgically dissected, the tissue fragments were dissociated into cell suspensions, cell
10 counts and viability were determined and the cells were transplanted either directly or after treatment with PT85 F(ab')₂ into the adult rat striatum. Rats were randomly assigned to one of four immunosuppression modalities: 1) no immunosuppression; 2) cyclosporin A (CyA) treatment only; 3) PT85 F(ab')₂ treatment only; or 4) cyclosporin A treatment and PT85 F(ab')₂ treatment. Four months after transplantation, the animals were sacrificed and the
15 brains were examined histologically for presence or absence of the grafts.

 PT85 F(ab')₂ fragments were generated using immobilized pepsin, as follows. Purified antibody was added, at 20 mg/ml in pH 4.7 digestion buffer and digested for 4.0 hours. The crude digest was removed from the pepsin and immediately neutralized with pH 7.0 binding buffer. The antibody mixture was applied to an immobilized Protein A column
20 and the elute was collected for the F(ab')₂ fragments. Dialysis against phosphate buffered saline for 24 h using 50,000 molecular weight cut-off tubing was then performed to rid the digest of contaminating Fc fragments. CHAPS buffer was added to the dialysis bag at a concentration of 10 mM. The completeness of the digest and purification of the F(ab')₂ were monitored by silver staining of 15% SDS polyacrylamide gels. Final purification of the
25 fragments was achieved by using a Superose 12 FPLC column. The completeness of Fc removal was demonstrated in an *in vitro* assay in which binding of the material to a target cell was followed with the addition of complement, and cytolysis of the pre-loaded target cells was measured by chromium release.

 PT85 F(ab')₂ treatment of the fetal porcine striatal cells prior to transplantation was
30 as follows: PT85 F(ab')₂ fragments prepared as described above were incubated with fetal porcine striatal cells at a dosage of about 1 µg of antibody per approximately 1 million cells. A solution of F(ab')₂ fragments at a concentration of about 10 µg/ml was used. Cells and antibody fragments were incubated for 30 minutes on ice, unbound antibody fragments were washed away and the cells were resuspended. The cells were then transplanted into the lesion
35 site in the recipient rats.

 Rats receiving cyclosporin A treatment were treated as follows: Cyclosporin A was administered subcutaneously in an olive oil vehicle at a dose of 8-10 mg/kg per day. Animals were kept on cyclosporin A for the duration of the experiment.

Data for graft survival from five different experiments are shown in Table 1.

TABLE 1

		No. Viable Cells		
5	Expt.	Transplanted/Rat	Immunosuppression	Graft Survival
	1	80,000	CyA	3/4
		80,000	F(ab') ₂	2/4
10		80,000	none	0/3
	2	150,000	CyA + F(ab') ₂	3/3
		150,000	F(ab') ₂	3/3
		150,000	none	0/5
15	3	200,000	CyA + F(ab') ₂	4/6
	.	200,000	CyA	6/7
	4	150,000	CyA + F(ab') ₂	6/7
20		150,000	CyA	3/6
		150,000	F(ab') ₂	3/8
	5	240,000	CyA	8/8
		240,000	F(ab') ₂	8/8
25				

EXAMPLE II: IMPLANTATION OF PORCINE HEPATOCYTES
IN A RABBIT MODEL OF HOMOZYGOUS FAMILIAL
HYPERCHOLESTEROLEMIA

The Watanabe Heritable Hyperlipidemic (WHHL) rabbit lacks functional low density lipoprotein (LDL) receptors and serves as an animal model for homozygous familial hypercholesterolemia (FH). This animal model is used herein to demonstrate the efficacy of xenogeneic transplantation as a means of providing LDL receptors and reducing serum cholesterol. The survival of such xenogeneic transplants is substantially prolonged when the donor cells are transplanted using the methodology described herein: masking of cell surface antigens prior to transplantation in conjunction with treatment of the recipient with a T cell inhibitory agent.

WHHL rabbits were purchased from CAMM Animal Facility, Wayne, N.J. Yorkshire outbred pigs (20-30 kg) were obtained from Tufts Veterinary Facility in Grafton, MA.

Yorkshire barrows were sacrificed and the left lateral lobe of the liver was mobilized, clamped and excised. The lobe was then perfused with cold PBS and reperfed with cold University of Wisconsin preservative (DuPont) for transport.

Hepatocytes were isolated from the liver lobe by the two stage perfusion technique originally described by Berry, M.N. and Friend, D.S. (1969) *J. Cell Biol.* 43:506-520 and modified by others (Maganto, P. et al. (1992) *Transplant. Proc.* 24:2826:2827; Gerlach, J.C. et al. (1994) *Transplantation* 57:1318-1322) for *ex vivo* perfusion of large animal organs. A liver lobe of 100-200 g was cannulated and perfused with Hanks Buffered Saline solution (HBSS) (minus Mg^{2+} and Ca^{2+}) containing 0.4 mM EDTA, 10 mM HEPES, pH 7.4 and penicillin (100 U/ml)- streptomycin (100 μ g/ml) at 35°C. This was followed by a second perfusion of complete HBSS containing collagenase P (0.8 mg/ml, Boehringer Mannheim) 10 mM HEPES, pH 7.4 and penicillin-streptomycin. The perfusion was continued until visible softening of the organ occurred. The total time for digestion ranged from 12-20 minutes. The digested liver lobe was then physically disrupted and the released hepatocytes were washed several times in DMEM/Weymouths media containing 10% heat inactivated calf serum at 4°C.

Hepatocytes were then collected and counted. Viability was assessed by trypan blue staining and was routinely greater than 90%. Cell yield varied from $1-8 \times 10^9$ hepatocytes per 150-200 g lobe. The purity of the hepatocyte preparation was judged to be over 98% by immunofluorescence for class II bearing non-parenchymal cells. Cells were stored in cold (4°C) complete HBSS and University of Wisconsin transport media prior to transplantation. Cells were transplanted within 5 hours of isolation.

$F(ab')_2$ fragments were prepared from the monoclonal antibody PT-85 specific for porcine MHC class I as described in Example 1. The purity of the preparation was assessed by SDS polyacrylamide gel electrophoresis with silver staining and found to be greater than 98% pure. FACS Scanning was utilized to determine the amount of $F(ab')_2$ fragments required to saturate all sites on hepatocytes by titration against a fixed number of cells in time periods of 0.5 to 2 hours. Titration of hepatocytes with anti-MHC antibodies yielded 50% saturation at 0.1 μ g of antibody per 10^6 cells. The expression of MHC class I on these cells was lower than found on porcine lymphocytes and endothelial cells, consistent with reports of MHC class I expression on hepatocytes. Bumgardner, G.L. et al. (1992) *Transplantation* 53:863-868. For double masking experiments, $F(ab')_2$ fragments of the monoclonal antibody PT85 were used in combination with $F(ab')_2$ fragments of the monoclonal antibody 9-3, which was generated against porcine MHC class I (Diacrin, Inc.). Specifically, monoclonal antibody 9-3 was raised against porcine lymphocytes in Balb/c mice. The mouse spleen cells were then fused with mouse myeloma cells using standard techniques and hybridomas were selected by reactivity with porcine endothelial cells and peripheral blood lymphocytes. The hybridoma was cloned by limiting dilution. The antibody was determined to be an IgG1-kappa. The monoclonal antibody 9-3 reacts with porcine MHC class I. The epitope for the

monoclonal antibody has been shown to be on the alpha-3 domain of MHC class I (the alpha-3 domain of porcine MHC class I is known-see, e.g., Satz, M.L. et al. (1985) *J. Immunol.* 135:2167-2175) and is separate from the epitope for the monoclonal antibody PT85. The procedure for generating F(ab')₂ fragments of the monoclonal antibody 9-3 was the same as
5 that for generating F(ab')₂ fragments of the monoclonal antibody PT85.

PT85 F(ab')₂ treatment of the porcine hepatocytes prior to transplantation was as follows: porcine hepatocytes isolated as described above were incubated with a ten fold excess of PT85 F(ab')₂ fragments prepared as described above (1 µg of antibody per
10 approximately 1 million cells) in PBS for one hour at 4°C. A solution of F(ab')₂ fragments at a concentration of about 10 µg/ml was used. For double masking experiments, porcine hepatocytes were incubated with a 10 fold excess of PT85 and 9-3 F(ab')₂ fragments in PBS for one hour at 4°C. Solutions of PT85 and 9-3 F(ab')₂ fragments at concentrations of about 10 µg/ml were used. As stated above, the monoclonal antibodies PT85 and 9-3 bind to
15 different epitopes on porcine MHC class I, as determined by standard binding competition assay.

Prior to transplantation, the hepatocytes were washed in Hanks solution at 4°C to remove unbound antibody fragments. WHHL rabbits (4-6 lbs) were anesthetized with xylazine and ketamine and maintained under isoflurane according to established protocols. The rabbits were then opened with a 5 cm incision distal and parallel to the end of the rib
20 cage. The peritoneum of each rabbit was incised and the portal veins exposed. Porcine hepatocytes suspended in 20 ml of HBSS containing penicillin/streptomycin or gentamycin and 10 units/ml heparin at 1×10^7 cells/ml at 37°C were infused into the portal veins via a 25 gauge syringe connected to a Harvard syringe pump at approximately 1 ml/min. The injection sites were covered with gel foam to prevent leakage. Using this general protocol,
25 surgical mortality was kept under 10%.

Survival and therapeutic effect of the transplanted hepatocytes were measured by assaying rabbit serum for porcine albumin and total serum cholesterol. The serum from the rabbits was subjected to immunoprecipitation using a species-specific anti-porcine albumin antibody (Research Plus) coupled to Sepharose 4B. Serum samples (50 µl) were diluted in a
30 phosphate buffered saline containing 0.2% Tween-20 (PBS-T) to a total reaction volume of 250 µl. Samples were incubated for 20 hours at 4°C with gentle rocking. Beads were centrifuged and washed in PBS-T prior to final resuspension in gel loading buffer. Samples were run on 8% SDS-PAGE. Gels were electrophoretically transferred to nitrocellulose and probed with a second species specific anti-porcine albumin antibody (Bethyl) Results were
35 visualized by ECL (Amersham). Pretransplant bleeds and commercial porcine albumin were used as controls.

Total rabbit serum cholesterol assays were performed as follows: pre and post operative rabbit serum was collected and analyzed for total serum cholesterol using Sigma (St. Louis, MO) diagnostic kit #352. Serum collections were made from non-fasted animals

using a morning bleed time for consistency. The serum was stored frozen and had undergone one round of freeze/thaw prior to assay. All serum samples were assayed at a 1:1 or 1:2 dilution with physiologic saline.

The following experiments were performed:

5
1) WHHL rabbits were transplanted with porcine hepatocytes double masked with 9-3 F(ab')₂ fragments and PT85 F(ab')₂ fragments, masked with PT85 F(ab')₂ fragments or unmasked porcine hepatocytes. These rabbits received cyclosporin A for two weeks. The percent serum cholesterol in serum from these rabbits over more than 60 days post
10 transplantation was measured. The results of this experiment are shown in Figure 1. Figure 1 demonstrates that transplantation of masked hepatocytes, when used in combination with cyclosporin treatment, resulted in reductions in serum cholesterol levels of over 20% for 6 weeks in 4 of 7 of rabbits with no return to pretransplant levels following cyclosporin A removal at two weeks. Of the three unsuccessful cases (R4046, R4053, R3775 (not shown)),
15 one showed no reduction in serum cholesterol and had no albumin secretion. In the other cases, cholesterol levels returned toward pretransplant values after several weeks. Absence of long term reductions in cholesterol in these cases are due to a less than 100% success of grafts. The grafts are judged unsuccessful if a reduction in cholesterol is not seen in a rabbit maintained on cyclosporin. If even one of these unsuccessful grafts is eliminated, the success
20 rate (defined as greater than 20% reduction in serum cholesterol for 6 weeks) for grafts in the rabbits receiving the combined masking and cyclosporin treatment is approximately 70%.

2) WHHL rabbits were transplanted with porcine hepatocytes masked with PT85 F(ab')₂ fragments or unmasked porcine hepatocytes. These rabbits received a single dose of
25 cyclosporin A on the day of surgery. The percent serum cholesterol in serum from these rabbits over more than 20 days post transplantation was measured. The results of this experiment are shown in Figures 2 and 3. Figure 2 demonstrates that rabbits transplanted with masked porcine hepatocytes and treated with a single dose of cyclosporin had lower serum cholesterol than rabbits transplanted with unmasked cells and treated with a single
30 dose of cyclosporin. Figure 3 shows that the masking/single dose cyclosporin combination treatment was more effective than masking without cyclosporin in the same experiment.

In sum, transplantation of masked porcine hepatocytes into the portal vein of WHHL rabbits, when combined with cyclosporin treatment, resulted in a decrease in serum
35 cholesterol in serum of these rabbits compared with serum cholesterol levels in serum from rabbits transplanted with unmasked hepatocytes and subject to cyclosporin treatment. The magnitude of the decrease was at least 20%. The grafts were successful in greater than 50% of the rabbits treated with cyclosporin A and continued to function for 60-100 days. The reduction in serum cholesterol is due to the LDL receptors provided by the transplanted cells.

The transplanted hepatocytes, visualized by immunohistochemistry of the rabbit livers, appeared to have integrated into the parenchyma to form a functional unit.

Other Embodiments

5 Other embodiments are within the scope of the invention and the following claims. For example, in another embodiment, cells which are administered to a subject according to the methods of the invention are present within a tissue or organ. When cells are within a tissue, antigens on the surface of the cells (e.g., MHC class I antigens) can be altered by contacting the entire tissue with a molecule (e.g., antibody) which binds to the antigen (e.g.,
10 incubating the tissue in a solution containing the molecule which binds the antigen). Alternatively, when a cell is within an organ, antigens on the surface of the cells (e.g., MHC class I antigens) can be altered by perfusing the organ with a solution containing a molecule (e.g., antibody) which binds to the antigen. An organ can be perfused with a solution containing the molecule using conventional techniques for organ perfusion.

15 In yet another embodiment, cells which are administered to a subject according to the methods of the invention are genetically modified to express a gene product. The genetically modified cells can be transplanted into a recipient subject to deliver the gene product to the subject. Cells can be genetically modified to express a gene product by introducing nucleic acid encoding the gene product into the cell. For example, a cell can be infected with a
20 recombinant virus (e.g., retrovirus, adenovirus) which contains the nucleic acid of interest. A non-human cell which is genetically modified to express a human gene product can be used to deliver the human gene product to a human subject by altering at least one antigen on the surface of the non-human cell and transplanting the cell into the recipient subject.

A cell can be modified to express a gene product by introducing genetic material, such as
25 a nucleic acid molecule (e.g., RNA or, more preferably, DNA) into the cell. The nucleic acid molecule introduced into the cell encodes a gene product to be expressed by the cell. The term "gene product" as used herein is intended to include proteins, peptides and functional RNA molecules. Generally, the gene product encoded by the nucleic acid molecule is the desired gene product to be supplied to a subject. Alternatively, the encoded gene product is
30 one which induces the expression of the desired gene product by the cell (e.g., the introduced genetic material encodes a transcription factor which induces the transcription of the gene product to be supplied to the subject).

A nucleic acid molecule introduced into a cell is in a form suitable for expression in
the cell of the gene product encoded by the nucleic acid. Accordingly, the nucleic acid
35 molecule includes coding and regulatory sequences required for transcription of a gene (or portion thereof) and, when the gene product is a protein or peptide, translation of the gene product encoded by the gene. Regulatory sequences which can be included in the nucleic acid molecule include promoters, enhancers and polyadenylation signals, as well as

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(21) International Application Number: PCT/US95/03959 (22) International Filing Date: 30 March 1995 (30.03.95) (30) Priority Data: 08/220,724 31 March 1994 (31.03.94) US (71) Applicant: DIACRIN, INC. [US/US]; Building 96, 13th Street, Charlestown Navy Yard, Charlestown, MA 02129 (US). (72) Inventor: FRASER, Thomas; 130 Franklin Street, Newton, MA 02158 (US). (74) Agents: SILVERI, Jean, M. et al.; Lahive & Cockfield, 60 State Street, Boston, MA 02109 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NI, PT, SE). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

(54) Title: IMPROVED METHODS FOR TRANSPLANTATION USING MODIFIED CELLS AND T CELL INHIBITORY AGENTS**(57) Abstract**

Improved methods for inhibiting rejection of transplanted cells in an allogeneic or xenogeneic recipient subject are described. The methods involve altering at least one antigen on the surface of a donor cell prior to transplantation to reduce the immunogenicity of the cell in a recipient subject. Preferably, an MHC class I antigen on a donor cell is altered by contacting the cell with a molecule which binds to the antigen, such as an antibody or fragment or derivative thereof. According to the methods of the invention, a recipient subject is also treated with an agent which inhibits T cell activity in the subject. A preferred agent which inhibits T cell activity in the recipient subject is an immunosuppressive drug, such as cyclosporin A. Alternatively, antibodies which deplete T cells or inhibit T cell proliferation in the subject can be used. Typically, following administration of a modified donor cell to an allogeneic or xenogeneic recipient subject, an immunosuppressive drug is administered to the recipient subject for a sufficient time to induce tolerance to the donor cells.

sequences necessary for transport of an encoded protein or peptide, for example N-terminal signal sequences for transport of proteins or peptides to the surface of the cell or for secretion.

Nucleotide sequences which regulate expression of a gene product (e.g., promoter and enhancer sequences) are selected based upon the type of cell in which the gene product is to be expressed and the desired level of expression of the gene product. For example, a promoter known to confer cell-type specific expression of a gene linked to the promoter can be used. A promoter specific for myoblast gene expression can be linked to a gene of interest to confer muscle-specific expression of that gene product. Muscle-specific regulatory elements which are known in the art include upstream regions from the dystrophin gene (Klamut et al., (1989) *Mol. Cell. Biol.* 9:2396), the creatine kinase gene (Buskin and Hauschka, (1989) *Mol. Cell Biol.* 9:2627) and the troponin gene (Mar and Ordahl, (1988) *Proc. Natl. Acad. Sci. USA.* 85:6404). Regulatory elements specific for other cell types are known in the art (e.g., the albumin enhancer for liver-specific expression; insulin regulatory elements for pancreatic islet cell-specific expression; various neural cell-specific regulatory elements, including neural dystrophin, neural enolase and A4 amyloid promoters). Alternatively, a regulatory element which can direct constitutive expression of a gene in a variety of different cell types, such as a viral regulatory element, can be used. Examples of viral promoters commonly used to drive gene expression include those derived from polyoma virus, Adenovirus 2, cytomegalovirus and Simian Virus 40, and retroviral LTRs. Alternatively, a regulatory element which provides inducible expression of a gene linked thereto can be used. The use of an inducible regulatory element (e.g., an inducible promoter) allows for modulation of the production of the gene product in the cell. Examples of potentially useful inducible regulatory systems for use in eukaryotic cells include hormone-regulated elements (e.g., see Mader, S. and White, J.H. (1993) *Proc. Natl. Acad. Sci. USA* 90:5603-5607), synthetic ligand-regulated elements (see, e.g. Spencer, D.M. et al. (1993) *Science* 262:1019-1024) and ionizing radiation-regulated elements (e.g., see Manome, Y. et al. (1993) *Biochemistry* 32:10607-10613; Datta, R. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10149-10153). Additional tissue-specific or inducible regulatory systems which may be developed can also be used in accordance with the invention.

There are a number of techniques known in the art for introducing genetic material into a cell that can be applied to modify a cell of the invention. In one embodiment, the nucleic acid is in the form of a naked nucleic acid molecule. In this situation, the nucleic acid molecule introduced into a cell to be modified consists only of the nucleic acid encoding the gene product and the necessary regulatory elements. Alternatively, the nucleic acid encoding the gene product (including the necessary regulatory elements) is contained within a plasmid vector. Examples of plasmid expression vectors include CDM8 (Seed, B., *Nature* 329:840 (1987)) and pMT2PC (Kaufman, et al., *EMBO J.* 6:187-195 (1987)). In another embodiment, the nucleic acid molecule to be introduced into a cell is contained within a viral vector. In this situation, the nucleic acid encoding the gene product is inserted into the viral

genome (or a partial viral genome). The regulatory elements directing the expression of the gene product can be included with the nucleic acid inserted into the viral genome (i.e., linked to the gene inserted into the viral genome) or can be provided by the viral genome itself. Examples of methods which can be used to introduce naked nucleic acid into cells and viral-mediated transfer of nucleic acid into cells are described separately in the subsections below.

A. Introduction of Naked Nucleic Acid into Cells

1. *Transfection mediated by CaPO_4* : Naked DNA can be introduced into cells by forming a precipitate containing the DNA and calcium phosphate. For example, a HEPES-buffered saline solution can be mixed with a solution containing calcium chloride and DNA to form a precipitate and the precipitate is then incubated with cells. A glycerol or dimethyl sulfoxide shock step can be added to increase the amount of DNA taken up by certain cells. CaPO_4 -mediated transfection can be used to stably (or transiently) transfect cells and is only applicable to *in vitro* modification of cells. Protocols for CaPO_4 -mediated transfection can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Section 9.1 and in Molecular Cloning: A Laboratory Manual, 2nd Edition, Sambrook et al. Cold Spring Harbor Laboratory Press, (1989), Sections 16.32-16.40 or other standard laboratory manuals.
2. *Transfection mediated by DEAE-dextran*: Naked DNA can be introduced into cells by forming a mixture of the DNA and DEAE-dextran and incubating the mixture with the cells. A dimethylsulfoxide or chloroquine shock step can be added to increase the amount of DNA uptake. DEAE-dextran transfection is only applicable to *in vitro* modification of cells and can be used to introduce DNA transiently into cells but is not preferred for creating stably transfected cells. Thus, this method can be used for short term production of a gene product but is not a method of choice for long-term production of a gene product. Protocols for DEAE-dextran-mediated transfection can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Section 9.2 and in Molecular Cloning: A Laboratory Manual, 2nd Edition, Sambrook et al. Cold Spring Harbor Laboratory Press, (1989), Sections 16.41-16.46 or other standard laboratory manuals.
3. *Electroporation*: Naked DNA can also be introduced into cells by incubating the cells and the DNA together in an appropriate buffer and subjecting the cells to a high-voltage electric pulse. The efficiency with which DNA is introduced into cells by electroporation is influenced by the strength of the applied field, the length of the electric pulse, the temperature, the conformation and concentration of the DNA and the ionic composition of the media. Electroporation can be used to stably (or transiently) transfect a wide variety of cell types and is only applicable to *in vitro* modification of cells. Protocols for

electroporating cells can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Section 9.3 and in Molecular Cloning: A Laboratory Manual, 2nd Edition, Sambrook et al. Cold Spring Harbor Laboratory Press, (1989), Sections 16.54-16.55 or other standard laboratory manuals.

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4. *Liposome-mediated transfection ("lipofection")*: Naked DNA can be introduced into cells by mixing the DNA with a liposome suspension containing cationic lipids. The DNA/liposome complex is then incubated with cells. Liposome mediated transfection can be used to stably (or transiently) transfect cells in culture *in vitro*. Protocols can be found in
10 Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Section 9.4 and other standard laboratory manuals. Additionally, gene delivery *in vivo* has been accomplished using liposomes. See for example Nicolau et al. (1987) *Meth. Enz.* 149:157-176; Wang and Huang (1987) *Proc. Natl. Acad. Sci. USA* 84:7851-7855; Brigham et al. (1989) *Am. J. Med. Sci.* 298:278; and Gould-Fogerite et al.
15 (1989) *Gene* 84:429-438.

5. *Direct Injection*: Naked DNA can be introduced into cells by directly injecting the DNA into the cells. For an *in vitro* culture of cells, DNA can be introduced by microinjection. Since each cell is microinjected individually, this approach is very labor intensive when
20 modifying large numbers of cells. However, a situation wherein microinjection is a method of choice is in the production of transgenic animals (discussed in greater detail below). In this situation, the DNA is stably introduced into a fertilized oocyte which is then allowed to develop into an animal. The resultant animal contains cells carrying the DNA introduced into the oocyte. Direct injection has also been used to introduce naked DNA into cells *in vivo* (see
25 e.g., Acsadi et al. (1991) *Nature* 332: 815-818; Wolff et al. (1990) *Science* 247:1465-1468). A delivery apparatus (e.g., a "gene gun") for injecting DNA into cells *in vivo* can be used. Such an apparatus is commercially available (e.g., from BioRad).

6. *Receptor-Mediated DNA Uptake*: Naked DNA can also be introduced into cells by
30 complexing the DNA to a cation, such as polylysine, which is coupled to a ligand for a cell-surface receptor (see for example Wu, G. and Wu, C.H. (1988) *J. Biol. Chem.* 263:14621; Wilson et al. (1992) *J. Biol. Chem.* 267:963-967; and U.S. Patent No. 5,166,320). Binding of the DNA-ligand complex to the receptor facilitates uptake of the DNA by receptor-mediated endocytosis. Receptors to which a DNA-ligand complex have targeted include the transferrin
35 receptor and the asialoglycoprotein receptor. A DNA-ligand complex linked to adenovirus capsids which naturally disrupt endosomes, thereby releasing material into the cytoplasm can be used to avoid degradation of the complex by intracellular lysosomes (see for example Curiel et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8850; Cristiano et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:2122-2126). Receptor-mediated DNA uptake can be used to introduce

DNA into cells either *in vitro* or *in vivo* and, additionally, has the added feature that DNA can be selectively targeted to a particular cell type by use of a ligand which binds to a receptor selectively expressed on a target cell of interest.

5 Generally, when naked DNA is introduced into cells in culture (e.g., by one of the transfection techniques described above) only a small fraction of cells (about 1 out of 10^5) typically integrate the transfected DNA into their genomes (i.e., the DNA is maintained in the cell episomally). Thus, in order to identify cells which have taken up exogenous DNA, it is advantageous to transfect nucleic acid encoding a selectable marker into the cell along with the nucleic acid(s) of interest. Preferred selectable markers include those which confer
10 resistance to drugs such as G418, hygromycin and methotrexate. Selectable markers may be introduced on the same plasmid as the gene(s) of interest or may be introduced on a separate plasmid.

An alternative method for generating a cell that is modified to express a gene product involving introducing naked DNA into cells is to create a transgenic animal which contains
15 cells modified to express the gene product of interest. A transgenic animal is an animal having cells that contain a transgene, wherein the transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA molecule which is integrated into the genome of a cell from which a transgenic animal develops and which remains in the genome of the mature animal, thereby directing the
20 expression of an encoded gene product in one or more cell types or tissues of the transgenic animal. Thus, a transgenic animal expressing a gene product of interest in one or more cell types within the animal can be created, for example, by introducing a nucleic acid encoding the gene product (typically linked to appropriate regulatory elements, such as a tissue-specific enhancer) into the male pronuclei of a fertilized oocyte, e.g., by microinjection, and allowing
25 the oocyte to develop in a pseudopregnant female foster animal. Methods for generating transgenic animals, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009 and Hogan, B. et al., (1986) A Laboratory Manual, Cold Spring Harbor, New York, Cold Spring Harbor Laboratory. A transgenic founder animal can be used to breed more animals carrying the
30 transgene. Cells of the transgenic animal which express a gene product of interest can then be used to deliver the gene product to a subject in accordance with the invention.

Alternatively, an animal containing a gene which has been modified by homologous recombination can be constructed to express a gene product of interest. For example, an endogenous gene carried in the genome of the animal can be altered by homologous
35 recombination (for instance, all or a portion of a gene could be replaced by the human homologue of the gene to "humanize" the gene product encoded by the gene) or an endogenous gene can be "knocked out" (i.e., inactivated by mutation). For example, an endogenous gene in a cell can be knocked out to prevent production of that gene product and then nucleic acid encoding a different (preferred) gene product is introduced into the cell. To

create an animal with homologously recombined nucleic acid, a vector is prepared which contains the DNA which is to replace or interrupt the endogenous DNA flanked by DNA homologous to the endogenous DNA (see for example Thomas, K.R. and Capecchi, M. R. (1987) *Cell* 51:503). The vector is introduced into an embryonal stem cell line (e.g., by electroporation) and cells which have homologously recombined the DNA are selected (see for example Li, E. et al. (1992) *Cell* 69:915). The selected cells are then injected into a blastocyst of an animal (e.g., a mouse) to form aggregation chimeras (see for example Bradley, A. in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed. (IRL, Oxford, 1987) pp. 113-152). A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term. Progeny harboring the homologously recombined DNA in their germ cells can be used to breed animals in which all cells of the animal contain the homologously recombined DNA. Cells of the animal containing the homologously recombined DNA which express a gene product of interest can then be used to deliver the gene product to a subject in accordance with the invention.

B. Viral-Mediated Gene Transfer

A preferred approach for introducing nucleic acid encoding a gene product into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding the gene product. Infection of cells with a viral vector has the advantage that a large proportion of cells receive the nucleic acid, which can obviate the need for selection of cells which have received the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells which have taken up viral vector nucleic acid and viral vector systems can be used either *in vitro* or *in vivo*.

1. *Retroviruses*: Defective retroviruses are well characterized for use in gene transfer for gene therapy purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). A recombinant retrovirus can be constructed having a nucleic acid encoding a gene product of interest inserted into the retroviral genome. Additionally, portions of the retroviral genome can be removed to render the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989), Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines include ψ Crip, ψ Cre, ψ 2 and ψ Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, *in*

- vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) *Science* 230:1395-1398; Danos and Mulligan (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-6464; Wilson et al. (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al. (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury et al. (1991) *Science* 254:1802-1805; van Beusechem et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay et al. (1992) *Human Gene Therapy* 3:641-647; Dai et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu et al. (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573). Retroviral vectors require target cell division in order for the retroviral genome (and foreign nucleic acid inserted into it) to be integrated into the host genome to stably introduce nucleic acid into the cell. Thus, it may be necessary to stimulate replication of the target cell.
2. **Adenoviruses:** The genome of an adenovirus can be manipulated such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See for example Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; and Rosenfeld et al. (1992) *Cell* 68:143-155. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses are advantageous in that they do not require dividing cells to be effective gene delivery vehicles and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld et al. (1992) cited *supra*), endothelial cells (Lemarchand et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:6482-6486), hepatocytes (Herz and Gerard (1993) *Proc. Natl. Acad. Sci. USA* 90:2812-2816) and muscle cells (Quantin et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584). Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al. cited *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors currently in use are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material.
3. **Adeno-Associated Viruses:** Adeno-associated virus (AAV) is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al. *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses

that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al. (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski et al. (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al. (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and
5 can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al. (1985) *Mol. Cell. Biol.* 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al. (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin et al. (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford et al. (1988)
10 *Mol. Endocrinol.* 2:32-39; Tratschin et al. (1984) *J. Virol.* 51:611-619; and Flotte et al. (1993) *J. Biol. Chem.* 268:3781-3790).

The efficacy of a particular expression vector system and method of introducing nucleic acid into a cell can be assessed by standard approaches routinely used in the art. For example, DNA introduced into a cell can be detected by a filter hybridization technique (e.g.,
15 Southern blotting) and RNA produced by transcription of introduced DNA can be detected, for example, by Northern blotting, RNase protection or reverse transcriptase-polymerase chain reaction (RT-PCR). The gene product can be detected by an appropriate assay, for example by immunological detection of a produced protein, such as with a specific antibody, or by a functional assay to detect a functional activity of the gene product, such as an
20 enzymatic assay. If the gene product of interest to be expressed by a cell is not readily assayable, an expression system can first be optimized using a reporter gene linked to the regulatory elements and vector to be used. The reporter gene encodes a gene product which is easily detectable and, thus, can be used to evaluate the efficacy of the system. Standard reporter genes used in the art include genes encoding β -galactosidase, chloramphenicol acetyl
25 transferase, luciferase and human growth hormone.

When the method used to introduce nucleic acid into a population of cells results in modification of a large proportion of the cells and efficient expression of the gene product by the cells (e.g., as is often the case when using a viral expression vector), the modified
30 population of cells may be used without further isolation or subcloning of individual cells within the population. That is, there may be sufficient production of the gene product by the population of cells such that no further cell isolation is needed. Alternatively, it may be desirable to grow a homogenous population of identically modified cells from a single modified cell to isolate cells which efficiently express the gene product. Such a population of
35 uniform cells can be prepared by isolating a single modified cell by limiting dilution cloning followed by expanding the single cell in culture into a clonal population of cells by standard techniques.

C. Other Methods for Modifying a Cell to Express a Gene Product

Alternative to introducing a nucleic acid molecule into a cell to modify the cell to express a gene product, a cell can be modified by inducing or increasing the level of expression of the gene product by a cell. For example, a cell may be capable of expressing a particular gene product but fails to do so without additional treatment of the cell. Similarly, the cell may express insufficient amounts of the gene product for the desired purpose. Thus, an agent which stimulates expression of a gene product can be used to induce or increase expression of a gene product by the cell. For example, cells can be contacted with an agent *in vitro* in a culture medium. The agent which stimulates expression of a gene product may function, for instance, by increasing transcription of the gene encoding the product, by increasing the rate of translation or stability (e.g., a post transcriptional modification such as a poly A tail) of an mRNA encoding the product or by increasing stability, transport or localization of the gene product. Examples of agents which can be used to induce expression of a gene product include cytokines and growth factors.

Another type of agent which can be used to induce or increase expression of a gene product by a cell is a transcription factor which upregulates transcription of the gene encoding the product. A transcription factor which upregulates the expression of a gene encoding a gene product of interest can be provided to a cell, for example, by introducing into the cell a nucleic acid molecule encoding the transcription factor. Thus, this approach represents an alternative type of nucleic acid molecule which can be introduced into the cell (for example by one of the previously discussed methods). In this case, the introduced nucleic acid does not directly encode the gene product of interest but rather causes production of the gene product by the cell indirectly by inducing expression of the gene product.

In yet another method, a cell is modified to express a gene product by coupling the gene product to the cell, preferably to the surface of the cell. For example, a protein can be obtained by purifying the cell from a biological source or expressing the protein recombinantly using standard recombinant DNA technology. The isolated protein can then be coupled to the cell. The terms "coupled" or "coupling" refer to a chemical, enzymatic or other means (e.g., by binding to an antibody on the surface of the cell or genetic engineering of linkages) by which a gene product can be linked to a cell such that the gene product is in a form suitable for delivering the gene product to a subject. For example, a protein can be chemically crosslinked to a cell surface using commercially available crosslinking reagents (Pierce, Rockford IL). Other approaches to coupling a gene product to a cell include the use of a bispecific antibody which binds both the gene product and a cell-surface molecule on the cell or modification of the gene product to include a lipophilic tail (e.g., by inositol phosphate linkage) which can insert into a cell membrane.

In yet another embodiment, a recipient subject into which altered cells of the invention are transplanted is also treated with a T cell inhibitory agent to further inhibit rejection of the transplanted cells. The T cell inhibitory agent inhibits T cell activity. For

example, the T cell inhibitory agent can be an immunosuppressive drug. A preferred immunosuppressive drug is cyclosporin A. Other immunosuppressive drugs which can be used include FK506 and RS-61443. Such immunosuppressive drugs can be used in conjunction with a steroid (e.g., glucocorticoids such as prednisone, methylprednisolone and dexamethasone) or chemotherapeutic agents (e.g., azathioprine and cyclophosphamide), or both. Alternatively, the T cell inhibitory agent can be one or more antibodies which deplete T cell activity, such as antibodies directed against T cell surface molecules (e.g., anti-CD2, anti-CD3, anti-CD4 and/or anti-CD8 antibodies).

10 EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method for transplanting a cell into an allogeneic or xenogeneic recipient subject such that rejection of the cell by the recipient subject is inhibited, comprising:
 - a) administering to the subject a cell having at least one antigen on the cell surface which stimulates an immune response against the cell in the recipient subject, wherein the at least one antigen on the cell surface is altered prior to transplantation; and
 - b) administering to the subject an agent which inhibits T cell activity in the subject.
2. The method of claim 1, wherein the antigen is altered to modify an interaction between the antigen and a T lymphocyte in the subject.
3. The method of claim 2, wherein the cell is contacted with at least one molecule which binds to the antigen.
4. The method of claim 3, wherein the at least one molecule which binds to the antigen is an antibody, or fragment or derivative thereof, which binds to the antigen but does not activate complement or induce lysis of the cell.
5. The method of claim 4, wherein the antibody, or fragment or derivative thereof, is an F(ab')₂ fragment.
6. The method of claim 2, wherein at least one antigen is an MHC class I antigen.
7. The method of claim 6, wherein the cell is contacted with at least one anti-MHC class I antibody, or fragment or derivative thereof, which binds to the MHC class I antigen but does not activate complement or induce lysis of the cell.
8. The method of claim 7, wherein the at least one anti-MHC class I antibody is an anti-MHC class I F(ab')₂ fragment.
9. The method of claim 8, wherein the anti-MHC class I F(ab')₂ fragment is a F(ab')₂ fragment of a monoclonal antibody selected from the group consisting of W6/32, PT85, a monoclonal antibody which binds an epitope bound by W6/32 and a monoclonal antibody which binds an epitope bound by PT85.

10. The method of claim 6, wherein the cell is contacted with at least one peptide which binds to an MHC class I antigen.

11. The method of claim 2, wherein the agent which inhibits T cell activity is an immunosuppressive drug.

12. The method of claim 2, wherein the agent which inhibits T cell activity is at least one antibody, or fragment or derivative thereof, which depletes T cells, sequesters T cells or inhibits T cell proliferation in the subject.

13. The method of claim 12, wherein the at least one antibody binds to a T cell surface molecule selected from the group consisting of CD2, CD3, CD4 and CD8.

14. The method of claim 2, wherein the cell is human.

15. The method of claim 2, wherein the cell is non-human.

16. A method for transplanting a cell into an allogeneic or xenogeneic recipient subject such that rejection of the cell by the recipient subject is inhibited, comprising:
a) administering to the subject a cell having at least one antigen on the cell surface which stimulates an immune response against the cell in the recipient subject, wherein the at least one antigen on the cell surface is altered prior to transplantation; and
b) administering to the subject an immunosuppressive drug.

17. The method of claim 16, wherein the antigen on the cell surface is altered to modify an interaction between the antigen and a T lymphocyte in the subject.

18. The method of claim 17, wherein the cell is contacted with at least one molecule which binds to the antigen.

19. The method of claim 18, wherein the at least one molecule which binds to the antigen is an antibody, or fragment or derivative thereof, which binds to the antigen but does not activate complement or induce lysis of the cell.

20. The method of claim 19, wherein the antibody, or fragment or derivative thereof, is an F(ab')₂ fragment.

21. The method of claim 17, wherein at least one antigen is an MHC class I antigen.

22. The method of claim 21, wherein the cell is contacted with at least one anti-MHC class I antibody, or fragment or derivative thereof, which binds to the MHC class I antigen but does not activate complement or induce lysis of the cell.

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23. The method of claim 22, wherein the at least one anti-MHC class I antibody is an anti-MHC class I F(ab')₂ fragment.

24. The method of claim 23, wherein the anti-MHC class I F(ab')₂ fragment is a F(ab')₂ fragment of a monoclonal antibody selected from the group consisting of W6/32, PT85, a monoclonal antibody which binds an epitope bound by W6/32 and a monoclonal antibody which binds an epitope bound by PT85.

10

25. The method of claim 21, wherein the cell is contacted with at least one peptide which binds to an MHC class I antigen.

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26. The method of claim 17, wherein the immunosuppressive drug is cyclosporin A.

27. The method of claim 17, wherein the immunosuppressive drug is FK506 or RS-61443.

20

28. The method of claim 17, wherein the immunosuppressive drug is administered in conjunction with a steroid or a chemotherapeutic agent.

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29. The method of claim 28, wherein the steroid is selected from the group consisting of prednisone, methylprednisolone and dexamethasone.

30. The method of claim 28, wherein the chemotherapeutic agent is selected from the group consisting of azathioprine and cyclophosphamide.

30

31. The method of claim 17, wherein the immunosuppressive drug is administered to the subject for a period of time sufficient to induce tolerance to the cell in the subject

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32. The method of claim 31, wherein the immunosuppressive drug is administered to the subject for not less than three days and not more than three months following administration of the cell.

33. The method of claim 31, wherein the immunosuppressive drug is administered to the subject for not less than one week and not more than one month following administration of the cell.

5 34. A method for transplanting a cell into an allogeneic or xenogeneic recipient subject such that rejection of the cell by the recipient subject is inhibited, comprising:

a) administering to the subject a cell having at least one MHC class I antigen on the cell surface which stimulates an immune response against the cell in the recipient subject, wherein the at least one MHC class I antigen on the cell surface is altered prior to
10 transplantation;

b) administering to the subject cyclosporin A

35. The method of claim 34, wherein the cell is contacted with at least one molecule which binds to an MHC class I antigen.
15

36. The method of claim 35, wherein a molecule which binds to the MHC class I antigen is an antibody, or fragment or derivative thereof, which binds to the antigen but does not activate complement or induce lysis of the cell.

20 37. The method of claim 36, wherein the antibody, or fragment or derivative thereof, is an F(ab')₂ fragment.

38. The method of claim 37, wherein the anti-MHC class I F(ab')₂ fragment is a F(ab')₂ fragment of a monoclonal antibody selected from the group consisting of W6/32, PT85, a monoclonal antibody which binds an epitope bound by W6/32 and a monoclonal
25 antibody which binds an epitope bound by PT85.

39. The method of claim 34, wherein the cell is contacted with at least one peptide which binds to an MHC class I antigen.
30

40. The method of claim 34, wherein the cyclosporine A is administered in conjunction with a steroid or a chemotherapeutic agent.

41. The method of claim 40, wherein the steroid is selected from the group
35 consisting of prednisone, methylprednisolone and dexamethasone.

42. The method of claim 40, wherein the chemotherapeutic agent is selected from the group consisting of azathioprine and cyclophosphamide.

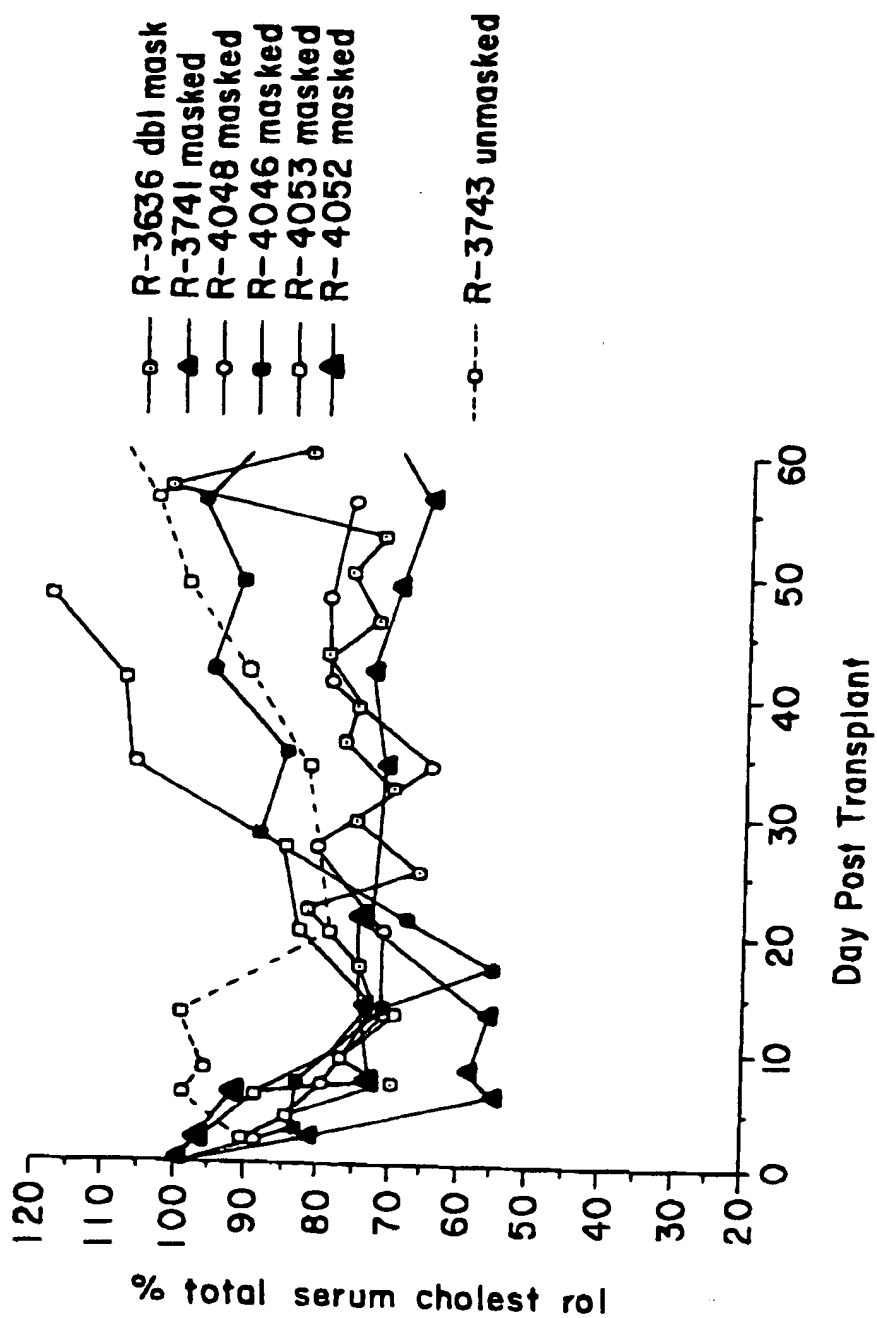
43. The method of claim 34, wherein the cyclosporine A is administered to the subject for a period of time sufficient to induce tolerance to the cell in the subject

5 44. The method of claim 34, wherein the cyclosporine A is administered to the subject for not less than three days and not more than three months following administration of the cell.

10 45. The method of claim 34, wherein the cyclosporine A is administered to the subject for not less than one week and not more than one month following administration of the cell.

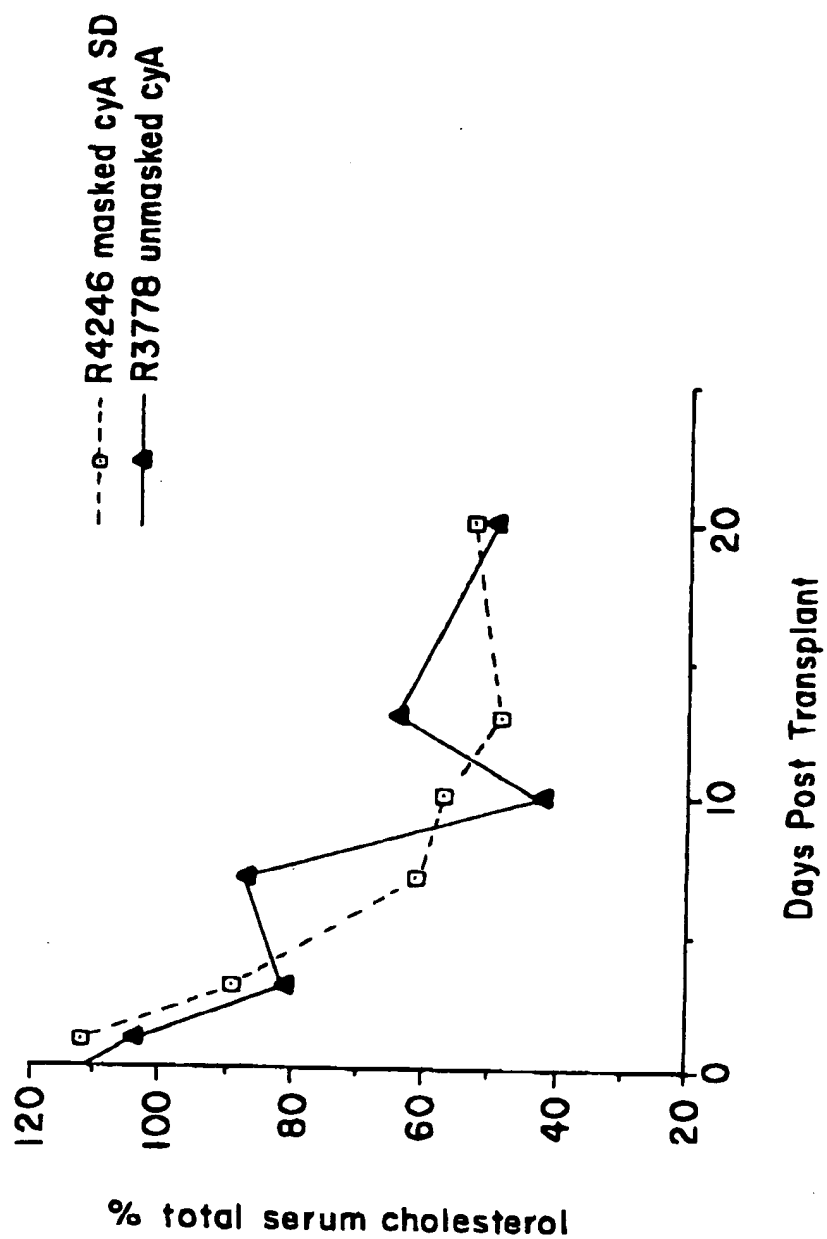
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FIG. 1



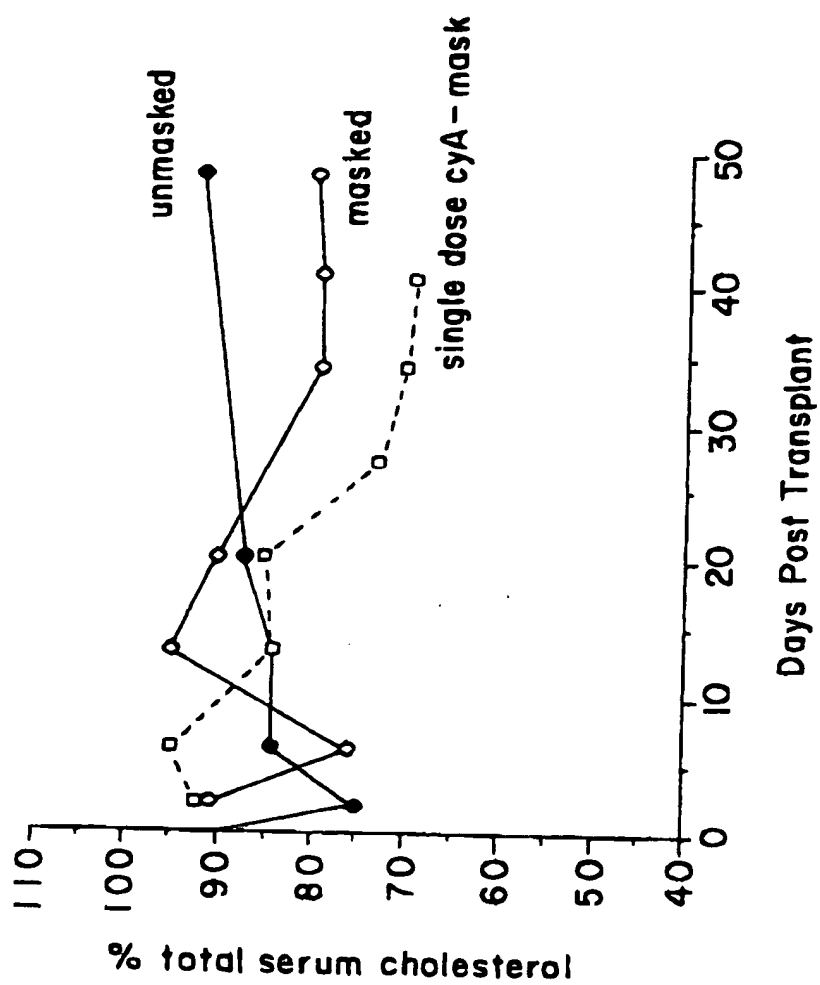
2 / 8

FIG. 2



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FIG. 3



SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/03959

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K35/39 A61K38/13 //(A61K38/13, 35:39), (A61K35/39, 31:445),
(A61K35/39, 31:52), (A61K35/39, 31:57), (A61K35/39, 31:675)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO-A-92 04033 (THE GENERAL HOSPITAL CORPORATION) 9 March 1992 cited in the application see examples see claims ---	1-9, 14, 16-24, 34-38
A	SCIENCE, vol. 252, no. 5013, 21 June 1991 WASHINGTON, DC, USA, pages 1700-1702, D. FAUSTMAN ET AL. 'Prevention of xenograft rejection by masking donor HLA class I antigens.' cited in the application see the whole document --- -/--	1-9, 14, 16-24, 34-38

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

11 August 1995

Date of mailing of the international search report

25. 08. 95

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/03959

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 90, no. 21, 1 November 1993 WASHINGTON, DC, USA, pages 9872-9876, J. GOSS ET AL. 'Specific prolongation of allograft survival by a T-cell-receptor-derived peptide.' cited in the application see abstract</p> <p>---</p>	1-3,6, 10, 16-18, 21,25, 34,35,39
A	<p>ADVANCES IN CANCER RESEARCH, vol. 52, 1989 SAN DIEGO, CA, USA, pages 151-163, S. PAXBO ET AL. 'Adenovirus proteins and MHC expression.' see the whole document</p> <p>---</p>	1,2,6, 16,17,34
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A	<p>WO-A-85 00954 (COLUMBUS UNIVERSITY) 14 March 1985</p> <p>see the whole document</p> <p>---</p>	1,2,6, 14,16, 17,21
P,X	<p>WO,A,94 16065 (EXEMPLAR CORPORATION) 21 July 1994</p> <p>see claims</p> <p>-----</p>	1,2,6, 11, 14-17, 21, 26-34, 40-45

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Information on patent family members

Intern. Appl. No.

PCT/US 95/03959

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